Exhibit 7

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Development of Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry Methods for Analysis of DNA Adducts of Formaldehyde and Their Application to Rats Treated with N-Nitrosodimethylamine or 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone

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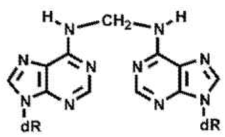
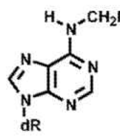
Reaction of formaldehyde with DNA in vitro produces a variety of adducts among which are observed the cross-link di-(N^6-deoxyadenosyl)methane (dAdo-CH_2-dAdo, **1**) and the hydroxymethyl adduct N^6-hydroxymethyl-dAdo (N^6-$HOCH_2$-dAdo, **2**). While the structures of these adducts have been known for decades, there have been no reports of their formation in vivo. Formaldehyde is released during intracellular metabolism of carcinogenic N-nitrosomethyl compounds such as N-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), but DNA adducts formed by this pathway have not been previously characterized. In this study, we addressed these questions by developing highly sensitive liquid chromatography–electrospray ionization–tandem mass spectrometry–selected reaction monitoring methods for quantitation of adducts **1** and **2**, the latter as N^6-methyl-dAdo (**3**). Considerable effort was devoted to the problem of artifactual formation of **1**, which can occur during storage of DNA samples by reaction of dAdo with **2**. This problem was solved by the addition of adenosine deaminase during the DNA hydrolysis step, thus removing dAdo as a reactant. The instability of adduct **2** was another potential block to analysis, and this was solved by converting it to **3** with $NaBH_3CN$. Separate aliquots of DNA were analyzed for adducts **1** and **2**, using the [^{15}N]-labeled adducts as internal standards. The application of these methods to rat hepatic DNA to which adducts **1** and **3** were added demonstrated accuracy and precision. The detection limits for adducts **1** and **3** were 1–4 adducts per 10^9 nucleotides using 100–150 μg of DNA. The method was applied to analyze hepatic and pulmonary DNA from rats treated with NDMA and NNK. The results clearly demonstrated the dose-dependent presence of N^6-$HOCH_2$-dAdo (**2**) in all DNA samples. The cross-link adduct dAdo-CH_2-dAdo (**1**) was observed in hepatic DNA of NNK-treated rats, with lower amounts in pulmonary DNA. Levels of these adducts were generally less than those of DNA adducts formed by the classical diazohydroxide pathway of nitrosamine metabolism. The results of this study demonstrate for the first time the presence of formaldehyde DNA adducts in tissues of rats treated with carcinogenic nitrosamines and suggest that these adducts may play a role in cancer induction by these compounds.

Introduction

Formaldehyde is described as "carcinogenic to humans" by the International Agency for Research on Cancer and "reasonably anticipated to be a human carcinogen" by the U.S. Department of Health and Human Services (*1, 2*). Formaldehyde produces squamous cell carcinomas of the nasal cavities in rats when administered by inhalation. Mixed results have been obtained using other routes of administration and animal species (*1, 2*). Formaldehyde is mutagenic in a variety of different test systems (*1*). DNA adducts are critical for mutagenicity and carcinogenicity. Several formaldehyde DNA adducts have been identified in vitro, notably the dAdo cross-link (dAdo-CH_2-dAdo, **1**) and its precursor N^6-hydroxymethyl-dAdo (N^6-$HOCH_2$-dAdo, **2**). Cross-links involving dGuo have also been identified as have other hydroxymethyl adducts with the exocyclic amino groups of dGuo and dCyd (*3–7*). At low formaldehyde concentrations in vitro, adduct **1** predominates (*7*). While extensive studies have characterized formaldehyde DNA–protein cross-links in vivo (*1*), there are no published data on the identification or quantitation of specific formaldehyde DNA adducts in either laboratory animals or humans. This is important because exposure to formaldehyde, an ubiquitous environmental agent and common metabolic product, is inevitable. In this paper, we describe the development of highly sensitive liquid chromatography–electrospray ionization–tandem mass spectrometry–selected reaction monitoring (LC-ESI-MS/MS-SRM) methods for analysis of formaldehyde DNA adducts **1** and **2**, with the latter being quantified as **3**, after $NaBH_3CN$ treatment.


dAdo-CH_2-dAdo, **1**


N^6-$HOCH_2$-dAdo, R=OH, **2**
N^6-CH_3-dAdo, R=H, **3**

dR=2'-deoxyribose

The methods were applied for the analysis of adducts **1** and **2** in rats treated with the carcinogenic nitrosamines N-ni-

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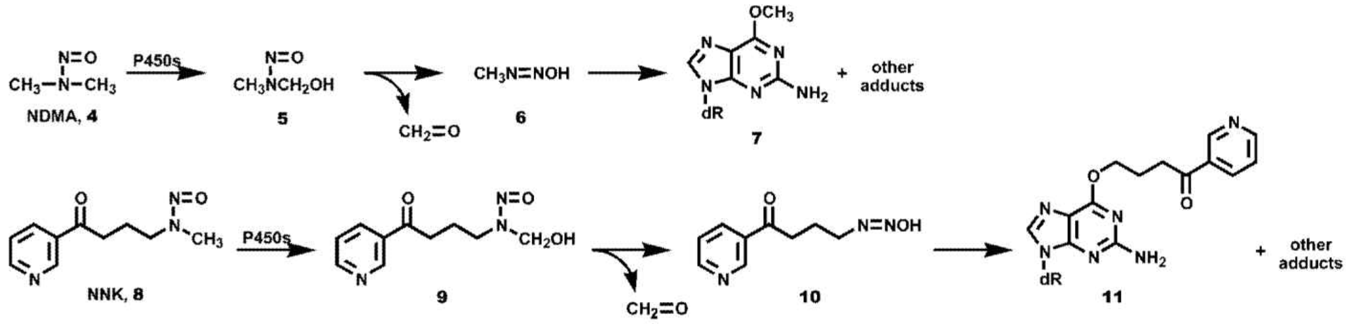
Scheme 1. Generation of Formaldehyde upon Methyl Hydroxylation of NDMA and NNK



trosodimethylamine (NDMA, **4**) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, **8**) (Scheme 1). NDMA and NNK are representative *N*-nitrosomethyl carcinogens (*8, 9*). Beginning with the landmark studies of Magee, Dutton, Heath, and Druckrey nearly 50 years ago, well-established pathways of metabolic activation of nitrosamines involving cytochrome P450-mediated α-methyl hydroxylation have been described in the literature (*8, 9*). As shown in Scheme 1, methyl hydroxylation of NDMA and NNK yields intermediates **5** and **9**, which spontaneously release reactive diazohydroxides **6** and **10**. These diazohydroxides or the corresponding diazonium ions react with DNA, producing adducts such as O^6-methyl-dGuo (**7**) from NDMA and O^6-pyridyloxobutyl-dGuo (O^6-POB-dGuo, **11**) from NNK. The roles in carcinogenesis of these and related methyl- and pyridyloxobutyl DNA adducts of NDMA, NNK, and other *N*-nitroso compounds have been extensively studied (*8–14*). Remarkably, however, there are no published data on DNA adduct formation by the other electrophilic product of α-methyl hydroxylation—formaldehyde. In this paper, we present the first evidence that formaldehyde DNA adducts are formed in the lung and liver of rats treated with NDMA and NNK.

Experimental Procedures

HPLC-UV Analysis. This was carried out using Waters Associates (Milford, MA) instruments equipped with a model 996 photodiode array detector for systems 1 and 2 or a Shimadzu SPD-10A UV detector (Shimadzu Scientific Instruments, Columbia, MD) for system 3. The UV detector was operated at 254 nm. System 1 used a 4.6 mm × 25 cm 5 μm Supelcosil LC 18-BD column (Supelco, Bellefonte, PA) with isocratic elution by 5% CH_3OH in 40 mM ammonium acetate buffer (pH 6.6) for 10 min and then a gradient from 5 to 35% CH_3OH over the course of 60 min at a flow rate of 0.5 mL/min. This system was used for the purification of dAdo-CH_2-dAdo, [$^{15}N_5$]dAdo-CH_2-dAdo, and N^6-Me-dAdo. System 2 used the same column as in system 1 with elution by a gradient from 40 to 60% CH_3OH in H_2O over the course of 40 min at a flow rate of 1 mL/min. It was used for desalting of isolated adducts. System 3 used a 4.6 mm × 25 cm Luna 5 μm C18 column (Phenomenex, Torrance, CA) with a gradient from 5 to 40 % CH_3OH over the course of 35 min at a flow rate of 0.7 mL/min. This system was used for quantitation of dGuo in enzymatic hydrolysates of DNA samples.

LC-ESI-MS/MS-SRM Analysis. The analyses of formaldehyde adducts were carried out with either a Finnigan Quantum Ultra AM (with Ion Max ESI source) or a Discovery Max triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA) interfaced with an Agilent 1100 capillary flow HPLC and a 150 mm × 0.5 mm Zorbax SB C18 column (Agilent Technologies, Palo Alto, CA). The column was operated at 25 °C. For analysis of N^6-Me-dAdo, we used isocratic elution by H_2O for 10 min, then a gradient to 25% CH_3CN over the course of 29 min, then 25–100% CH_3CN for 2 min, then 100% CH_3CN for 10 min, and finally returning to H_2O in 3 min, at a flow rate of 15 μL/min. For analysis of dAdo-CH_2-dAdo, we used isocratic elution by 5% CH_3OH in H_2O for 10 min, then a gradient from 5 to 40% CH_3OH over the course of 5 min, then isocratic elution by 40% CH_3OH for 15 min, then 40–100% CH_3OH in 1 min, then 100% CH_3OH for 10 min, and finally returning to 5% CH_3OH in 5 min, at a flow rate of 15 μL/min. For both adducts, the first 20 min of eluant was directed to waste, and the 20–30 min fractions were diverted to the ESI source. The MS parameters were set as follows: spray voltage, 4 kV; sheath gas pressure, 40; capillary temperature, 250 °C; collision energy, 15 V; scan width, 0.1 amu; scan time, 0.1 s; Q1 peak width, 0.7 amu; Q3 peak width, 0.7; Q2 pressure, 1.0 mTorr; source CID, 10 V; and tube lens offset, 70 V. Transitions monitored were as follows: dAdo-CH_2-dAdo (**1**) and [$^{15}N_5$]dAdo-CH_2-[$^{15}N_5$]dAdo ([$^{15}N_5$]**1**) *m/z* 515 $[M + H]^+ \rightarrow$ *m/z* 252 $[dAdo + H]^+$ and *m/z* 525 → *m/z* 257; N^6-Me-dAdo (**3**) and [$^{15}N_5$]N^6-Me-dAdo ([$^{15}N_5$]**3**) *m/z* 266 $[M + H]^+ \rightarrow$ *m/z* 150 $[BH]^+$ and *m/z* 271 → *m/z* 155.

Chemicals and Enzymes. [$^{15}N_5$]dAdo was obtained from Spectra Stable Isotopes (Columbia, MD). Ethanol was obtained from AAPER Alcohol and Chemical Co. (Shelbyville, KY). 2-Propanol was purchased from Acros Organics (Morris Plains, NJ). Puregene DNA purification solution was procured from Gentra Systems (Minneapolis, MN). Alkaline phosphatase was obtained from Roche Diagnostics Corp. (Indianapolis, IN). DNA adduct standards were obtained as described (*15*). N^6-Me-dAdo and all other chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO).

dAdo-CH_2-dAdo (1) and [$^{15}N_5$]dAdo-CH_2-[$^{15}N_5$]dAdo ([$^{15}N_5$]1). dAdo-CH_2-dAdo (**1**) was prepared as described from the reaction of dAdo and formaldehyde (*7*), except that the procedure for purification was modified. In brief, dAdo (60 mg, 0.4 mmol) was allowed to react with formaldehyde (200 mg, 6.6 mol, 500 μL of 37% in H_2O) in 12 mL of 0.1 M phosphate buffer (pH 7.0) at 37 °C for 264 h. A C18 Sep-Pak (Waters, 35 mL, 10 g) was conditioned with 20 mL portions of CH_3OH, H_2O and 0.1 M phosphate buffer (pH 7.0). The reaction mixture was applied, and the column was eluted with 20 mL portions of 0.1 M phosphate buffer (pH 7.0), 20% CH_3OH, 50% CH_3OH, and 100% CH_3OH. The fraction eluting with 50% CH_3OH contained dAdo-CH_2-dAdo. It was dried using a SpeedVac, dissolved in 200 μL of H_2O, and purified using HPLC systems 1 and 2, to give 10 mg of dAdo-CH_2-dAdo, 5% yield. UV, λ_{max} 272 nm, lit (*3*) 272 nm. ESI-MS *m/z* 537 $[M + Na]^+$ (rel int 76), 515 $[M + H]^+$ (100), 252 $[dAdo + H]^+$ (63). Purity 99%, HPLC system 1.

[$^{15}N_5$]dAdo-CH_2-[$^{15}N_5$]dAdo ([$^{15}N_5$]**1**) was prepared the same way from [$^{15}N_5$]dAdo and was identified using LC-ESI-MS/MS-SRM at *m/z* 525 → *m/z* 257 as compared to *m/z* 515 → *m/z* 252 for dAdo-CH_2-dAdo and by UV, λ_{max} 271 nm. ESI-MS *m/z* 547 $[M + Na]^+$ (rel int 63), 525 $[M + H]^+$ (100), 257 $[dAdo + H]^+$ (66). Purity 99%, HPLC System 1. The concentration of the standard solution was determined by comparison to the dAdo-CH_2-dAdo calibration curve using LC-ESI-MS/MS-SRM.

[$^{15}N_5$]N^6-Me-dAdo ([$^{15}N_5$]3). This was prepared essentially as described (*16*). A mixture of [$^{15}N_5$]dAdo (15 mg, 0.06 mmol) and CH_3I (35 mg, 0.24 mmol) in *N,N*-dimethylacetamide (50 μL) was stirred overnight at room temperature in a tightly sealed flask. Acetone (250 μL) was added, and stirring was continued for 30 min. The solvent was removed under reduced pressure at room

temperature to give crude [$^{15}N_5$]1-methyl-dAdo iodide (20 mg). This was heated in 500 μL of 0.2 N NaOH for 30 min in a steam bath (causing rearrangement to [$^{15}N_5$]**3**) (*16*) and then cooled to room temperature. The resulting solution was adjusted to pH 7 with 10% aqueous *p*-toluenesulfonic acid. The solvents were removed under reduced pressure, and the residue was dissolved in 1 mL of H_2O and purified by HPLC systems 1 and 2 to give [$^{15}N_5$]N^6-Me-dAdo. UV, λ_{max} 265, 211 nm. ESI-MS *m/z* 293 [M + Na]$^+$ (rel int 5), 271 [M + H]$^+$ (100), 155 [BH]$^+$(18). Purity 97%, HPLC system 1. The concentration of the standard solution was determined by comparison to the N^6-Me-dAdo calibration curve using LC-ESI-MS/MS-SRM.

Treatment of Rats with NDMA or NNK. Male F344 rats were purchased from Charles River Laboratories (Wilmington, MA) and housed under standard conditions in the Research Animal Resources facility of the University of Minnesota. They were maintained on a NIH-07 diet (Harlan, Madison, WI) and tap water and were divided into five groups, each consisting of five rats (225–275 g body weight). The rats were each given s.c. injections of either 0.4 mL of saline (control) or 0.4 mL of saline containing 1.85 mg (0.025 mmol)/kg b.w. NDMA or 7.4 mg (0.1 mmol)/kg b.w. NDMA, or 5.2 mg (0.025 mmol)/kg b.w. NNK or 20.7 mg (0.1 mmol)/kg b.w. NNK. Injections were given once daily for four consecutive days. Four hours after the final injection, the rats were killed by CO_2 overdose. The liver and lung were harvested and stored at −80 °C. The doses were based on previous studies of DNA adduct detection in rats treated with these compounds (*17, 18*) and on a comparative study of NDMA and NNK carcinogenesis in rats in which the rats were treated with 0.0055 mmol/kg of each compound by s.c. injection, three times weekly for 20 weeks (*19*).

DNA Isolation. DNA isolation from rat liver and lung was performed as described (*20*). For experiments using $NaBH_3CN$ and [^{13}C]formaldehyde (Cambridge Isotope Laboratories, Cambridge, MA) to investigate artifact formation, 100 ppm of [^{13}C]formaldehyde was added to the cell lysis solution before the tissue was homogenized. The DNA was isolated from one aliquot of the tissue as usual, while in a second aliquot, 6.28 mg/mL of $NaBH_3CN$ was added to the cell lysis solution, the protein precipitation solution, and the other solutions and reagents used in the DNA isolation procedure: 2-propanol, Tris-EDTA buffer, ethanol, and 70% ethanol.

Analysis of DNA for N^6-HOCH$_2$-dAdo (2), as N^6-Me-dAdo (3). The procedure for enzymatic hydrolysis of DNA was previously described (*20*). In brief, DNA (0.1–0.3 mg) was dissolved in 500 μL of 10 mM Tris-HCl/5 mM $MgCl_2$ buffer (pH 7.0) containing [$^{15}N_5$]N^6-Me-dAdo (42.5 fmol) and $NaBH_3CN$ (30 mg). The DNA was initially digested overnight at room temperature with 530 units of DNase I (type II, bovine pancreas). The pH was adjusted to 7 with 1 N HCl, and to the resulting mixture were added 530 additional units of DNase I, 0.05 units of phosphodiesterase I (type II, *Crotalus adamanteus* venom), and 300 units of alkaline phosphatase (calf intestine). The mixture was incubated at 37 °C for 60 min. Enzymes were removed by centrifugation using a Centrifree MPS device, MW cut off 30000 (amico-ym 30000, Amicon, Beverly, MA). The hydrolysate, after removal of a 10 μL aliquot for dGuo quantitation, was desalted and purified using a solid phase extraction cartridge (SPE, Strata-X 33 μm, 30 mg/1 mL, Phenomenex). The cartridge was conditioned with two 1 mL portions of CH_3OH and 1 mL of H_2O. The sample was loaded, and the column was washed with 1 mL of H_2O and 1 mL of 10% CH_3OH in H_2O, which were discarded. It was then washed with 2 mL of 100% CH_3OH. The 100% CH_3OH fraction was collected in 4 mL silane-treated autosampler vials (Chrom Tech, Inc., Apple Valley, MN) and evaporated to dryness on a SpeedVac. The residue was dissolved in 20 μL of H_2O, and 8 μL aliquots were analyzed by LC-ESI-MS/MS-SRM.

Analysis of DNA for dAdo-CH$_2$-dAdo (1). A separate aliquot of DNA (0.1–0.3 mg) was used for this analysis, as adduct **1** is not stable to $NaBH_3CN$ treatment. For enzymatic hydrolysis of DNA, the procedure was the same as above with the following exceptions: $NaBH_3CN$ was not used; [$^{15}N_5$]dAdo-CH$_2$-[$^{15}N_5$]dAdo (50 fmol) was added as internal standard instead of [$^{15}N_5$]N^6-Me-dAdo; adenosine deaminase (4 units) was added at the beginning of the hydrolysis procedure; and the amount of DNase I was 1060 units. The amount of adenosine deaminase was sufficient to convert all dAdo to deoxyinosine. The purification of the hydrolysate was also the same as described above, except that 20% CH_3OH instead of 10% CH_3OH was used in the SPE. dAdo-CH$_2$-dAdo was eluted from the cartridge with 100% CH_3OH.

Table 1. Effect of Adenosine Deaminase on Measurement of dAdo-CH$_2$-dAdo in Salmon Testes DNA[a]

sample	adenosine deaminase	dAdo-CH$_2$-dAdo (fmol) 0 h[a]	72 h	1 week
1	not present	2.6	20 (8)[b]	64 (25)
2	not present	0.5	4.3 (9)	47 (94)
3	present	2.2	1.6 (0.7)	1.7 (0.8)
4	present	2.5	2.3 (1)	2.9 (1.2)

[a] DNA samples (1 mg) were enzymatically hydrolyzed in the absence or presence of adenosine deaminase as described in the Experimental Procedures. Samples were then stored at −20 °C for 72 h or 1 week prior to analysis by LC-ESI-MS/MS-SRM. All samples were analyzed in one batch. [b] Fold increase as compared to 0 h.

Analysis of DNA for POB–DNA Adducts. These were analyzed as previously described (*15*).

Results

Investigation of Artifact Formation in the Analysis of dAdo-CH$_2$-dAdo (1) in DNA. In our initial experiments, we observed substantial increases (4–100-fold) in measured levels of dAdo-CH$_2$-dAdo in DNA samples from livers of NDMA-treated rats, when these samples were isolated without SPE and were stored at −20 °C or room temperature for 2 days. We investigated the origin of this apparent artifact.

A likely possible explanation was the reaction of dAdo with N^6-HOCH$_2$-dAdo during workup or sample storage. DNA hydrolysates from tissues of rats exposed to formaldehyde could contain—in addition to dAdo—N^6-HOCH$_2$-dAdo, dAdo-CH$_2$-dAdo, and other potential formaldehyde DNA adducts. Previous studies have shown that dAdo reacts with N^6-HOCH$_2$-dAdo to produce dAdo-CH$_2$-dAdo (*3, 4*). It seemed likely that this could be the source of the increased dAdo-CH$_2$-dAdo levels found upon sample storage. We used SPE on a Strata-X cartridge to separate dAdo and N^6-HOCH$_2$-dAdo (which both eluted with 20% aqueous CH_3OH) from dAdo-CH$_2$-dAdo (which eluted with 100% CH_3OH). Although this separation method was not 100% efficient, as discussed below, we found that there was no increase in levels of dAdo-CH$_2$-dAdo when the samples in which this procedure was used were stored for 24 h. However, there was a 15-fold increase in dAdo-CH$_2$-dAdo levels when the Strata-X cartridge was omitted. These results indicated that the increased amount of dAdo-CH$_2$-dAdo observed in our stored samples resulted from further reactions of dAdo with N^6-HOCH$_2$-dAdo during storage.

One way to avoid this artifactual formation of dAdo-CH$_2$-dAdo would be to destroy one of the reactants—dAdo. Therefore, we investigated the use of adenosine deaminase for this purpose. Samples of salmon testes DNA, which we had determined to contain dAdo-CH$_2$-dAdo (data not shown), were enzymatically hydrolyzed and analyzed by LC-ESI-MS/MS-SRM under the conditions described below. As shown in Table 1, levels of dAdo-CH$_2$-dAdo did not change upon storage of these samples when adenosine deaminase was included in the enzymatic hydrolysis procedure, but the amounts did increase substantially when adenosine deaminase was omitted from the enzymatic hydrolysis mixture, and the samples were then stored. Further experiments demonstrated that dAdo-CH$_2$-dAdo could

Table 2. Investigation of Artifactual Formation of dAdo-CH_2-dAdo from Formaldehyde during Isolation and Hydrolysis of DNA from Rat Liver[a]

rat liver sample	addition of $NaBH_3CN$	fmol/mg DNA: dAdo-CH_2-dAdo	fmol/mg DNA: dAdo-[^{13}C]CH_2-dAdo
A	all steps	14	not detected
B	none	15	31

[a] [^{13}C]Formaldehyde (100 ppm) was added following cell lysis, and the enzymatic hydrolysis of DNA and analysis for the adducts were carried out by LC-ESI-MS/MS-SRM as described in the Experimental Procedures, except that adenosine deaminase was not used.

also form during preparation of samples for LC-ESI-MS/MS analysis if adenosine deaminase was omitted from the enzymatic hydrolysis, even if SPE was included, because SPE was not 100% efficient in the removal of dAdo and N^6-$HOCH_2$-dAdo from the samples. We also showed that dAdo-CH_2-dAdo was stable in the presence of adenosine deaminase, as long as excess DNA or dAdo was present. However, in the absence of DNA or dAdo, dAdo-CH_2-dAdo was degraded by adenosine deaminase. The amounts and timing of adenosine deaminase treatment required for prevention of artifact formation without degradation of dAdo-CH_2-dAdo were investigated to establish the conditions described in the Experimental Procedures.

A second possible source of artifact formation would be the release of formaldehyde from tissues or cells during DNA isolation. To test this possibility, we added [^{13}C]formaldehyde to the cell lysis solution used in the isolation of DNA from rat liver and then carried out the remaining steps in the DNA isolation and enzymatic hydrolysis procedure either in the absence or in the presence of $NaBH_3CN$. As summarized in Table 2, artifactual formation of dAdo-CH_2-dAdo can occur, because we did detect dAdo-[^{13}C]CH_2-dAdo when $NaBH_3CN$ was omitted. However, the amounts of isotopically normal dAdo-CH_2-dAdo in these two samples were the same, demonstrating that artifact formation did not occur from formaldehyde released during DNA isolation and enzymatic hydrolysis, because if it had there would have been a corresponding increase in the amount of dAdo-CH_2-dAdo.

A third possible source of artifact formation would be the reagents used in the analysis. These may have contained trace amounts of formaldehyde that could have reacted with dAdo in the samples. Therefore, we incubated various concentrations of dAdo (from 3.1 to 62.5 μg/mL) at 37 °C, in H_2O, CH_3OH, Tris-HCl buffer, and aqueous or buffer solutions of the enzymes, for up to 48 h, and then analyzed them for dAdo-CH_2-dAdo. The results of these experiments indicated that there was no formation of this adduct under any of these conditions.

Method for the Analysis of dAdo-CH_2-dAdo (1) in DNA. The analytical method is summarized in Figure 1. [$^{15}N_5$]dAdo-CH_2-[$^{15}N_5$]dAdo was added to the DNA samples as internal standard, and adenosine deaminase was included for the reasons described above. After enzyme hydrolysis of the DNA, high molecular weight material was removed by filtration, and the hydrolysate was partially purified by SPE. Analysis was carried out by LC-ESI-MS/MS-SRM, monitoring at m/z 515 $[M + H]^+ \rightarrow m/z$ 252 $[dAdo + H]^+$ for dAdo-CH_2-dAdo and m/z 525 $\rightarrow m/z$ 257 for the internal standard. Typical chromatograms obtained upon analysis of dAdo-CH_2-dAdo in hepatic DNA from an NDMA-treated and an untreated rat are illustrated in Figure 2. A clear peak, which coeluted with the internal standard, was observed in the treated rat sample but not in the control. The limit of detection of dAdo-CH_2-dAdo was 25 amol ($S/N = 4$) on column and approximately 1 adduct per 10^9 nucleotides using a 100 μg DNA sample. Analyte recovery was approximately 65%. The method was tested by adding various amounts of dAdo-CH_2-dAdo to rat hepatic DNA. As shown in Table 3, there was excellent agreement between the added and the detected amounts, demonstrating the accuracy of the method. Coefficients of variation (CV) were less than 10% (Table 3).

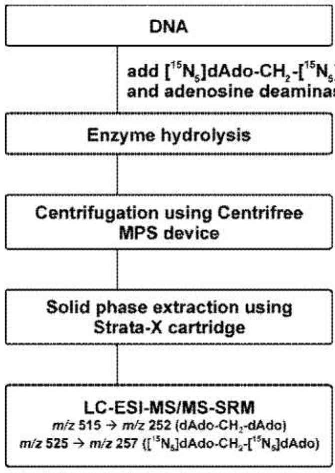


Figure 1. Outline of analytical method for determination of dAdo-CH_2-dAdo in DNA.

Aspects of Method Development for N^6-$HOCH_2$-dAdo (2), as N^6-Me-dAdo (3), in DNA. Because N^6-$HOCH_2$-dAdo is somewhat unstable (*4*), we developed conditions to convert it to N^6-Me-dAdo by $NaBH_3CN$ reduction. Various amounts of $NaBH_3CN$, reaction times, and temperatures were investigated to establish the conditions described in the Experimental Procedures. In salmon testes DNA, N^6-$HOCH_2$-dAdo was converted to N^6-Me-dAdo under these conditions, in an estimated 50–80% yield. Some N^6-Me-dAdo (6–8% of the total after reduction) was present in salmon testes DNA prior to $NaBH_3CN$ treatment. We also found that dAdo-CH_2-dAdo was unstable under these conditions. Thus, separate aliquots of DNA were required for analysis of N^6-$HOCH_2$-dAdo (as N^6-Me-dAdo) and dAdo-CH_2-dAdo.

Method for the Analysis of N^6-$HOCH_2$-dAdo, as N^6-Me-dAdo, in DNA. The method for analysis of N^6-$HOCH_2$-dAdo is similar to that outlined in Figure 1 for dAdo-CH_2-dAdo, except that the internal standard was [$^{15}N_5$]N^6-Me-dAdo, adenosine deaminase was not added, and $NaBH_3CN$ was included in the enzymatic hydrolysis mixture to convert N^6-$HOCH_2$-dAdo to N^6-Me-dAdo. Analysis was carried out by LC-ESI-MS/MS-SRM, monitoring at m/z 266 $[M + H]^+ \rightarrow m/z$ 150 $[BH]^+$ for N^6-Me-dAdo and m/z 271 $\rightarrow m/z$ 155 for the internal standard. Typical chromatograms obtained upon analysis of N^6-$HOCH_2$-dAdo, as N^6-Me-dAdo, in pulmonary DNA from an NNK-treated and from an untreated rat are illustrated in Figure 3. Clear peaks corresponding to N^6-Me-dAdo and coeluting with the internal standard were observed in both the treated and the control rat DNA samples. The detection limit of N^6-Me-dAdo was 200 amol ($S/N = 3$) on column and approximately 4 adducts per 10^9 nucleotides starting with 150 μg of DNA. Recovery of analyte was greater than 90%. The method was tested by addition of various amounts of N^6-Me-dAdo to rat hepatic DNA. There was excellent agreement between added and detected amounts, as shown in Table 4, and the CVs were less than 10% except at the lowest concentration.

Quantitation of Adducts in Hepatic and Pulmonary DNA from NDMA and NNK-Treated Rats. As indicated

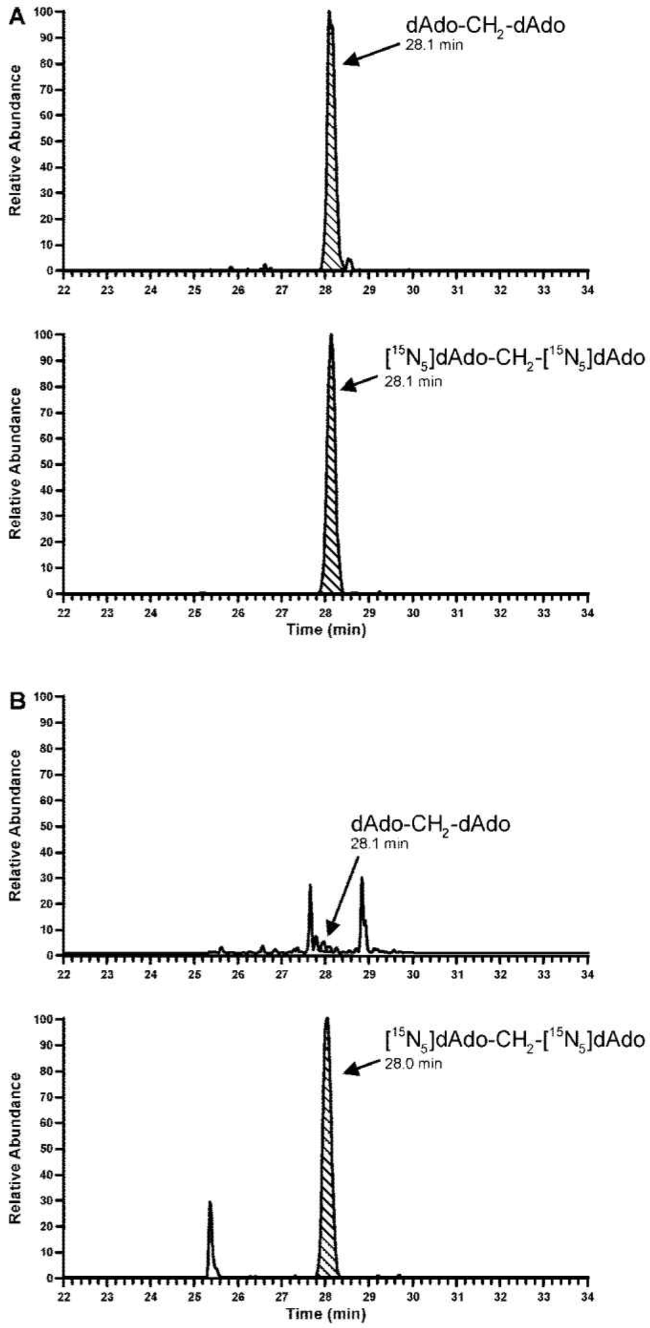


Figure 2. LC-ESI-MS/MS-SRM chromatogram of dAdo-CH_2-dAdo from hepatic DNA of an NDMA-treated rat (A) and from a control rat (B). Areas of the dAdo-CH_2-dAdo peak ($\times 10^4$): (A) 7.0 and (B) 0.03. The arrow in B points to the small dAdo-CH_2-dAdo peak.

Table 3. Analysis of Rat Hepatic DNA to Which dAdo-CH_2-dAdo Was Added

dAdo-CH_2-dAdo (fmol)[a]		
added	detected[b]	CV (%)
10	9.2 ± 0.7	7
15	14 ± 0.9	6
20	20 ± 1.4	0.7
25	25 ± 1.3	0.5
30	30 ± 1.1	0.4

[a] Added to 0.25 mg of rat hepatic DNA. [b] Mean $\pm$ SD ($N = 3$).

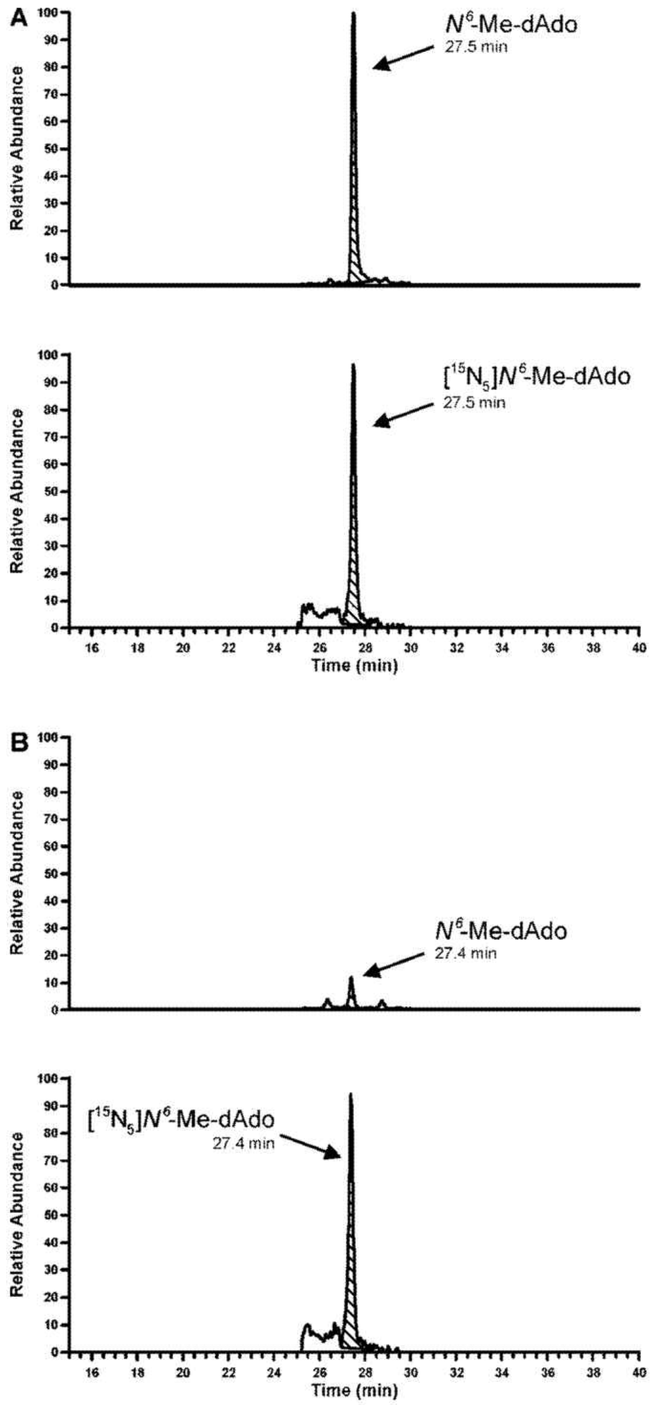


Figure 3. LC-ESI-MS/MS-SRM chromatogram of N^6-Me-dAdo from $NaBH_3CN$-treated pulmonary DNA of an NNK-treated rat (A) and from a control rat (B). Areas of the N^6-Me-dAdo peak ($\times 10^6$): (A) 5.0 and (B) 0.5.

above, clean chromatograms were obtained upon analysis by LC-ESI-MS/MS-SRM for dAdo-CH_2-dAdo and N^6-$HOCH_2$-dAdo in DNA of NDMA and NNK-treated rats (Figures 2 and 3). We also carried out qualitative LC-ESI-MS-SIM experiments with hepatic DNA from NDMA-treated rats, which further support the identity of the adducts. A chromatogram obtained upon LC-ESI-MS-SIM analysis (m/z 282) of hepatic DNA from an NDMA-treated rat is shown in Figure 4A. Peaks corresponding to the retention times of standard 7-Me-dGuo, N^6-$HOCH_2$-dAdo, and O^6-Me-dGuo were observed. The peak eluting at 47.0 min had a base peak at m/z 282 $[M + H]^+$ and a peak at m/z 166 $[BH]^+$, similar to standard N^6-$HOCH_2$-dAdo. Treatment with $NaBH_3CN$ of hepatic DNA from an NDMA-treated rat caused disappearance of the N^6-$HOCH_2$-dAdo peak and appearance of the corresponding peak for N^6-Me-dAdo (data not shown). LC-ESI-MS-SIM analysis (m/z 515) showed a peak at

Table 4. Analysis of Rat Hepatic DNA to Which N^6-Me-dA Was Added

N^6-Me-dAdo (fmol)[a,b]		
added	detected[c]	CV (%)
10	11 ± 1.9	18
20	21 ± 2.0	9
40	38 ± 0.3	1
80	74 ± 1.8	2
160	150 ± 6.1	4

[a] Added to 0.25 mg of rat hepatic DNA. [b] N^6-Me-dAdo in $NaBH_3$-CN-treated rat liver DNA (8.3 fmol/0.25 mg) was subtracted from each value. [c] Mean ± SD ($N = 3$).

85.8 min, corresponding to the retention time of dAdo-CH_2-dAdo (Figure 4B).

Adduct levels in liver and lung of NDMA-treated rats are summarized in Table 5. N^6-$HOCH_2$-dA (as N^6-Me-dAdo) was detected in both lung and liver, with higher amounts in liver. Levels of N^6-$HOCH_2$-dAdo increased with dose. N^6-$HOCH_2$-dAdo was also detected in DNA from control animals but at lower levels than in the NDMA-treated rats. Levels of N^6-$HOCH_2$-dAdo were less than those of O^6-methyl-dGuo in both liver and lung (data not shown). Levels of dAdo-CH_2-dAdo were near the detection limit in livers of the NDMA-treated rats.

Adduct levels in the liver and lung of NNK-treated rats are presented in Table 6. As in the NDMA-treated rats, N^6-$HOCH_2$-dAdo was detected in both lung and liver, with higher amounts in the liver. Levels of N^6-$HOCH_2$-dAdo increased with dose. Amounts of N^6-$HOCH_2$-dAdo were lower than all pyridyloxobutyl (POB) adducts of NNK except O^6-POB-dGuo. dAdo-CH_2-dAdo was clearly detected in liver DNA of NNK-treated rats, in amounts greater than in the lung or in control animals. The amounts of dAdo-CH_2-dAdo in the lung were near the detection limit. Levels of dAdo-CH_2-dAdo were lower than those of all POB adducts in the liver except O^6-POB-dGuo at the higher dose.

There was considerable rat to rat variation in the amounts of the formaldehyde adducts. This was not due to analytical variation, as demonstrated in Tables 3 and 4, and was greater than the inter-rat variation in levels of the other adducts measured.

Discussion

The results of this study provide the first evidence for the presence of formaldehyde DNA adducts in laboratory animals. We developed reliable and precise LC-ESI-MS/MS-SRM methods for the quantitative analysis of N^6-$HOCH_2$-dAdo (as N^6-Me-dAdo) and dAdo-CH_2-dAdo, two adducts known to result from the reaction of formaldehyde with DNA. Adduct identities were supported by the specific transitions monitored and by the appearance of clear, symmetric peaks at the same retention times as the [^{15}N]-labeled internal standards. The method was applied to rats treated with the carcinogenic nitrosamines NDMA and NNK, and the results demonstrate for the first time that formaldehyde DNA adducts are produced from these carcinogens, in addition to the well-characterized adducts, which result from diazohydroxides formed in nitrosamine metabolism.

The early literature on formaldehyde DNA adducts has been reviewed by Singer and Grunberger (*21*), Feldman (*22*), and Auerbach et al. (*23*). Shapiro and co-workers were the first to characterize cross-link adducts of formaldehyde, including dAdo-CH_2-dAdo, in RNA and DNA (*3*). Beland et al. fully characterized hydroxymethyl adducts of formaldehyde in reactions with deoxyribonucleosides and detected N^6-$HOCH_2$-dAdo in CHO cells treated with millimolar concentrations of labeled formaldehyde (*4*). Huang and Hopkins found that cross-links were preferentially formed in 5′-d(AT) sequences and characterized these as dAdo-CH_2-dAdo (*5, 6*), consistent with the earlier results of McGhee and von Hippel (*24–27*). We also observed the preferential formation of dAdo-CH_2-dAdo in reactions of DNA with concentrations of formaldehyde and acetaldehyde as low as 1 μM (*7*). Xhong and Hee detected hydroxymethyl adducts in human placental DNA reacted with 3.3 mM formaldehyde, using HPLC with UV, fluorescence, and electrochemical detection (*28*), and in human nasal epithelial cells after in vitro exposure to high levels of formaldehyde (*29*). The methods described here are substantially more sensitive than those reported previously and logically extend these earlier studies by demonstrating the presence of N^6-$HOCH_2$-dAdo and dAdo-CH_2-dAdo in vivo in rats.

The observation of formaldehyde adducts in DNA of rats treated with NDMA and NNK potentially creates a new dimension in our understanding of the mechanisms by which these nitrosamines induce cancer. Considerable evidence supports the role of persistent O^6-methyl-dGuo as a necessary but possibly not sufficient factor in carcinogenesis by NDMA and other methylating carcinogens (*8, 10*) and in lung carcinogenesis by NNK in the A/J mouse (*30*). Persistent mutagenic POB DNA and methyl DNA adducts are clearly important in rat lung carcinogenesis by NNK (*9, 14, 31*). The formaldehyde adducts detected here demonstrate that NDMA and NNK are indeed "bident carcinogens", a term introduced by Loeppky and co-workers to describe DNA alkylation by both carbocations and aldehydes generated from nitrosamines such as *N*-nitrosodiethanolamine and related compounds (*32*). While the levels of dAdo-CH_2-dAdo in liver of NDMA-treated rats and lung of NNK-treated rats were sometimes near the detection limit of our assays, there is no doubt that dAdo-CH_2-dAdo was formed in a dose-dependent manner in rat liver upon treatment with NNK. Cross-links such as dAdo-CH_2-dAdo are potentially one of the most serious types of DNA damage, as they could inhibit DNA strand separation required for replication, transcription, and recombination, but cells have developed efficient repair systems to remove such adducts (*33*). While no data are available specifically on dAdo-CH_2-dAdo, we note that its levels and distribution in NNK-treated rats do not correlate with the ability of NNK to induce lung tumors in rats (*19*). However, NNK and NDMA also induced liver tumors in rats when given at total doses comparable to those used here (*19*), and further studies are clearly needed to assess the formation and persistence of dAdo-CH_2-dAdo under the conditions of lung tumor induction by NNK in rats. Levels of N^6-$HOCH_2$-dA were dose-dependent in both liver and lung of NDMA- and NNK-treated rats, and they are clearly related to nitrosamine treatment. No information is available on the biological properties of this adduct, but it is known to be a precursor to dAdo-CH_2-dAdo (*3*) and could also serve as a source of DNA–protein cross-links, which are proposed to be important in formaldehyde-induced carcinogenesis (*1*). N^6-$HOCH_2$-dAdo could also be a source of intracellular formaldehyde generation, which has been observed to be a factor in cell proliferation and cell death (*34*). The reactivity of N^6-$HOCH_2$-dAdo in DNA might also explain the inter-rat variability observed in the analysis of this adduct. As formaldehyde generation is common in the metabolism of many noncarcinogenic compounds, it seems most likely that the role of formaldehyde DNA adducts in nitrosamine carcinogenesis would be best viewed as a potential adjunct to those

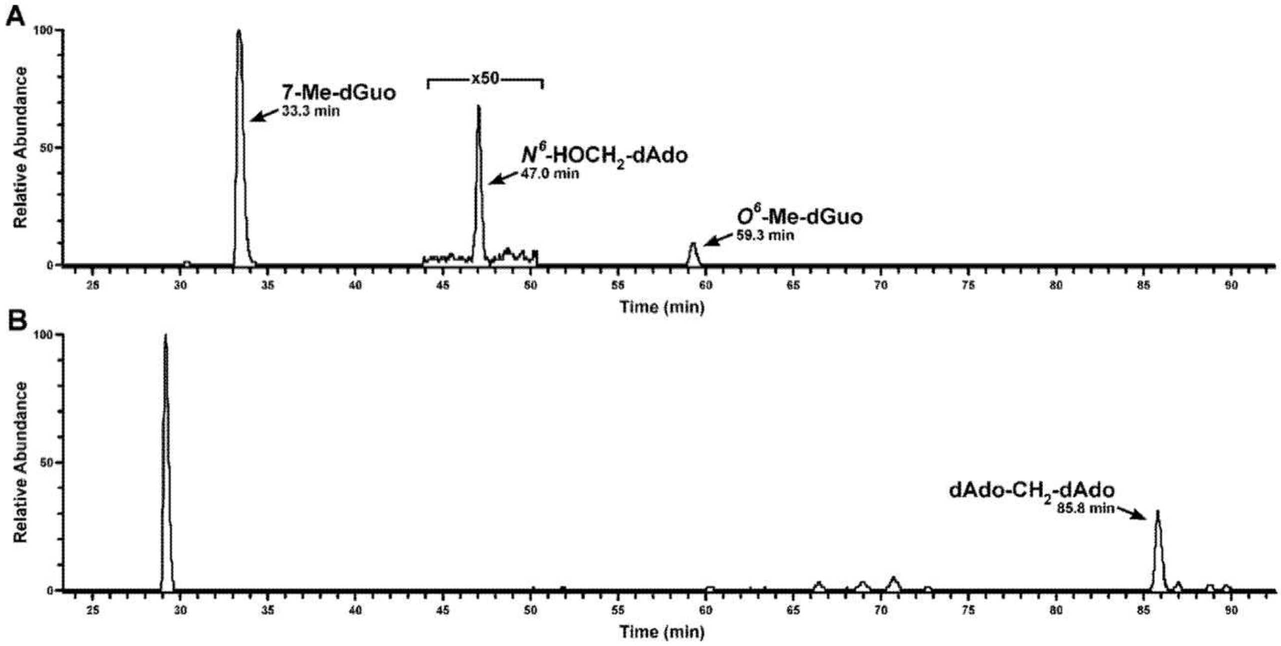


Figure 4. LC-ESI-MS-SIM analysis of an enzymatic hydrolysate of hepatic DNA from a rat treated with NDMA: (A) *m/z* 282 and (B) *m/z* 515.

Table 5. Adduct Levels in NDMA-Treated Rats

tissue	dose of NDMA (mmol/kg)[a]	mean ± SD, $N = 5$ (fmol/mg DNA) N^6-HOCH$_2$-dAdo	dAdo-CH$_2$-dAdo
lung	0.025	192 ± 48	not done
	0.1	319 ± 70	not done
	saline control	84 ± 7	not done
liver	0.025	596 ± 124	7 ± 4[b]
	0.1	1980 ± 604	5 ± 2[c]
	saline control	133 ± 126	9[d]

[a] Administered by s.c. injection daily for 4 days. [b] dAdo-CH$_2$-dAdo was not detected in two samples; the number shown is the mean of three. [c] dAdo-CH$_2$-dAdo was not detected in one sample; the number shown is the mean of four. [d] dAdo-CH$_2$-dAdo was not detected in three samples; the number shown is the mean of two.

formed by the diazohydroxide pathway, rather than having sufficient biological activity themselves.

It is also clear from our results that N^6-HOCH$_2$-dAdo, and possibly dAdo-CH$_2$-dAdo, is an endogenous DNA adduct in rats, as substantial amounts were detected in hepatic DNA of saline-treated animals. This is logical because formaldehyde is an essential metabolic intermediate in cells, produced endogenously from serine, glycine, methionine, and choline among other cellular constituents (*1*). It would also be logical that active repair systems exist to remove formaldehyde DNA adducts, but this requires further study.

The methods described here should be useful in examining a number of questions pertinent to the role of formaldehyde in carcinogenesis. As mentioned above, it will be important to assess the formation and persistence of formaldehyde DNA adducts vs diazohydroxide DNA adducts under the precise conditions of tumor induction by *N*-nitrosomethyl carcinogens. Another pressing question is the mechanism of carcinogenesis of formaldehyde itself, which induces nasal tumors when administered to rats by inhalation (*1*). Formaldehyde DNA protein cross-links as well as compensatory prolifieration have been proposed as mechanisms, but the role of DNA adducts has not been investigated, in spite of the known genotoxicity of formaldehyde (*1*). Formaldehyde is a metabolite of numerous pharmaceutical agents as well as nicotine and caffeine and is also released in aspartame metabolism, but little information is available on DNA adduct formation from these compounds (*35*). Formaldehyde DNA adducts could also serve as biomarkers for human exposure. Formaldehyde is rated as carcinogenic to humans by the IARC, causing nasopharyngeal cancer, while the data for its possible role as a cause of leukemia are considered equivocal (*1*). The methods described here may be useful in further examining the role of formaldehyde in human cancer.

A limitation of this study is that we do not know the extent of conversion of N^6-HOCH$_2$-dAdo to N^6-Me-dAdo in DNA, although we estimate that it is about 50–80%. Because our internal standard is [^{15}N$_5$]N^6-Me-dAdo, this potential loss of analyte is not taken into account. We could not use [^{15}N$_5$]N^6-HOCH$_2$-dAdo, or DNA containing this, as an internal standard

Table 6. Adduct Levels in NNK-Treated Rats

tissue	dose of NNK (mmol/kg)[a]	mean ± SD, $N = 5$[b] (fmol/mg DNA) N^6-HOCH$_2$-dAdo	dAdo-CH$_2$-dAdo	7-POB-Gua	O^2-POB-dThd	O^2-POB-Cyt	O^6-POB-dGuo
lung	0.025	230 ± 84	2[c]	933 ± 89	1120 ± 66	483 ± 36	251 ± 26
	0.1	615 ± 504	18[c]	1800 ± 478	2020 ± 483	840 ± 169	487 ± 101
	saline control	84 ± 7	2[d]	ND[g]	ND	ND	ND
liver	0.025	586 ± 191[e]	17 ± 12[e,f]	3550 ± 1600	3530 ± 725	2930 ± 521	28 ± 17
	0.1	3720 ± 2210	303 ± 290	12200 ± 1600	12300 ± 1690	7800 ± 1680	140 ± 25
	saline control	133 ± 126	9[c]	ND	ND	ND	ND

[a] Administered by s.c. injection daily for 4 days. [b] Except where noted otherwise. [c] dAdo-CH$_2$-dAdo was not detected in three samples; the number shown is the mean of two. [d] $N = 2$. [e] $N = 4$. [f] dAdo-CH$_2$-dAdo was not detected in one sample; the number shown is the mean of three. [g] ND, not detected (detection limit, 3 fmol/mg DNA).

because of its instability. Thus, the data in Tables 5 and 6 may underestimate the actual levels of N^6-HOCH$_2$-dAdo in the DNA samples.

In summary, we describe LC-ESI-MS/MS-SRM methods for quantitation of the formaldehyde DNA adducts N^6-HOCH$_2$-dAdo and dAdo-CH$_2$-dAdo in vivo. Using these methods, we demonstrated that these adducts are formed in the metabolism of the carcinogenic nitrosamines NDMA and NNK, indicating that, in addition to the well-established diazohydroxide pathway of nitrosamine carcinogenesis, released formaldehyde also contributes to DNA adduct formation and possibly to cancer induction by these compounds.

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References

(1) International Agency for Research on Cancer (2006) Formaldehyde, 2-butoxyethanol and 1-tert-butoxypropan-2-ol. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, Vol. 88, pp 39–325, IARC, Lyon, France.

(2) U.S. Department of Health and Human Services (2004) *11th Report on Carcinogens*, U.S. Government Printing Office, Washington, DC.

(3) Chaw, Y. F., Crane, L. E., Lange, P., and Shapiro, R. (1980) Isolation and identification of cross-links from formaldehyde-treated nucleic acids. *Biochemistry 19*, 5525–5531.

(4) Beland, F. A., Fullerton, N. F., and Heflich, R. H. (1984) Rapid isolation, hydrolysis and chromatography of formaldehyde-modified DNA. *J. Chromatogr. 308*, 121–131.

(5) Huang, H., and Hopkins, P. B. (1993) DNA interstrand cross-linking by formaldehyde: nucleotide sequence preference and covalent structure of the predominant cross-link formed in synthetic oligonucleotides. *J. Am. Chem. Soc. 115*, 9402–9408.

(6) Huang, H., Solomon, M. S., and Hopkins, P. B. (1992) Formaldehyde preferentially interstrand cross-links duplex DNA through deoxyguanosine residues at the sequence 5′-d(AT). *J. Am. Chem. Soc. 114*, 9240–9241.

(7) Cheng, G., Shi, Y., Sturla, S., Jalas, J., McIntee, E. J., Villalta, P. W., Wang, M., and Hecht, S. S. (2003) Reactions of formaldehyde plus acetaldehyde with deoxyguanosine and DNA: formation of cyclic deoxyguanosine adducts and formaldehyde cross-links. *Chem. Res. Toxicol. 16*, 145–152.

(8) Preussmann,R., and Stewart, B. W. (1984) *N*-Nitroso carcinogens. In *Chemical Carcinogens*, 2nd ed., (Searle,C. E., Ed.) ACS Monograph 182, Vol. 2, pp 643–828, American Chemical Society, Washington, DC.

(9) Hecht, S. S. (1998) Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. *Chem. Res. Toxicol. 11*, 559–603.

(10) Shuker, D. E., and Bartsch, H. (1994) DNA adducts of nitrosamines. In *IARC Sci. Publ. No. 125. DNA Adducts: Identification and Biological Significance* (Hemminki, K.,Dipple, A., Shuker, D. E. G. , Kadlubar, F. F. , Segerback, D., and Bartsch, H., Eds.) pp 73–89, Lyon, France.

(11) Kyrtopoulos, S. A., Anderson, L. M., Chhabra, S. K., Souliotis, V. L., Pletsa, V., Valanis, C., and Georgiadis, P. (1997) DNA adducts and the mechanism of carcinogenesis and cytotoxicity of methylating agents of environmental and clinical significance. *Cancer Detect. Prevention 21*, 391–405.

(12) Choi, J. Y., Chowdhury, G., Zang, H., Angel, K. C., Vu, C. C., Peterson, L. A., and Guengerich, F. P. (2006) Translesion synthesis across O^6-alkylguanine DNA adducts by recombinant human DNA polymerases. *J. Biol. Chem. 281*, 38244–38256.

(13) Lao, Y., Yu, N., Kassie, F., Villalta, P. W., and Hecht, S. S. (2007) Analysis of pyridyloxobutyl DNA adducts in F344 rats chronically treated with (*R*)- and (*S*)-*N*′-nitrosonornicotine. *Chem. Res. Toxicol. 20*, 246–256.

(14) Lao, Y., Yu, N., Kassie, F., Villalta, P. W., and Hecht, S. S. (2007) Formation and accumulation of pyridyloxobutyl DNA adducts in F344 rats chronically treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and enantiomers of its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. *Chem. Res. Toxicol. 20*, 235–245.

(15) Lao, Y., Villalta, P. W., Sturla, S. J., Wang, M., and Hecht, S. S. (2006) Quantitation of pyridyloxobutyl DNA adducts of tobacco-specific nitrosamines in rat tissue DNA by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry. *Chem. Res. Toxicol. 19*, 674–682.

(16) Jones, J. W., and Robins, R. K. (1963) Purine nucleosides. III. Methylation studies of certain naturally occurring purine nucleosides. *J. Am. Chem. Soc. 85*, 193–201.

(17) Murphy, S. E., Palomino, A., Hecht, S. S., and Hoffmann, D. (1990) Dose-response study of DNA and hemoglobin adduct formation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in F344 rats. *Cancer Res. 50*, 5446–5452.

(18) Devereux, T. R., Anderson, M. W., and Belinsky, S. A. (1988) Factors regulating activation and DNA alkylation by 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone and nitrosodimethylamine in rat lung and isolated lung cells, and their relationship to carcinogenicity. *Cancer Res. 48*, 4215–4221.

(19) Hecht, S. S., Trushin, N., Castonguay, A., and Rivenson, A. (1986) Comparative tumorigenicity and DNA methylation in F344 rats by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N-nitrosodimethylamine. *Cancer Res. 46*, 498–502.

(20) Wang, M., Yu, N., Chen, L., Villalta, P. W., Hochalter, J. B., and Hecht, S. S. (2006) Identification of an acetaldehyde adduct in human liver DNA and quantitation as N^2-ethyldeoxyguanosine. *Chem. Res. Toxicol. 19*, 319–324.

(21) Singer, B., and Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, pp 45–96, Plenum Press, New York.

(22) Feldman, M. Y. (1973) Reactions of nucleic acids and nucleoproteins with formaldehyde. *Prog. Nucleic Acid. Res. Mol. Biol. 13*, 1–49.

(23) Auerbach, C., Moutschen-Dahmen, M., and Moutschen, J. (1977) Genetic and cytogenetical effects of formaldehyde and related compounds. *Mutat. Res. 39*, 317–361.

(24) McGhee, J. D., and von Hippel, P. H. (1975) Formaldehyde as a probe of DNA structure. I. Reaction with exocyclic amino groups of DNA bases. *Biochemistry 14*, 1281–1296.

(25) McGhee, J. D., and von Hippel, P. H. (1975) Formaldehyde as a probe of DNA structure. II. Reaction with endocyclic imino groups of DNA bases. *Biochemistry 14*, 1297–1303.

(26) McGhee, J. D., and von Hippel, P. H. (1977) Formaldehyde as a probe of DNA structure. 3. Equilibrium denaturation of DNA and synthetic polynucleotides. *Biochemistry 16*, 3267–3276.

(27) McGhee, J. D., and von Hippel, P. H. (1977) Formaldehyde as a probe of DNA structure. Mechanism of the initial reaction of formaldehyde with DNA. *Biochemistry 16*, 3276–3293.

(28) Zhong, W., and Hee, S. S. (2005) Comparison of UV, fluorescence, and electrochemical detectors for the analysis of formaldehyde-induced DNA adducts. *J. Anal. Toxicol. 29*, 182–187.

(29) Zhong, W., and Que Hee, S. S. (2004) Formaldehyde-induced DNA adducts as biomarkers of in vitro human nasal epithelial cell exposure to formaldehyde. *Mutat. Res. 563*, 13–24.

(30) Peterson, L. A., and Hecht, S. S. (1991) O^6-Methylguanine is a critical determinant of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone tumorigenesis in A/J mouse lung. *Cancer Res. 51*, 5557–5564.

(31) Pauly, G. T., Peterson, L. A., and Moschel, R. C. (2002) Mutagenesis by O^6-[4-oxo-4-(3-pyridyl)butyl]guanine] in *Escherichia coli* and human cells. *Chem. Res. Toxicol. 15*, 165–169.

(32) Loeppky, R. N., and Goelzer, P. (2002) Microsome-mediated oxidation of N-nitrosodiethanolamine (NDELA), a bident carcinogen. *Chem. Res. Toxicol. 15*, 457–469.

(33) Liu, X., Lao, Y., Yang, I.-Y., Hecht, S. S., and Moriya, M. (2006) Replication-coupled repair of crotonaldehyde/acetaldehyde induced guanine-guanine interstrand cross-links and their mutagenicity. *Biochemistry 45*, 12898–12905.

(34) Nudelman, A., Levovich, I., Cutts, S. M., Phillips, D. R., and Rephaeli, A. (2005) The role of intracellularly released formaldehyde and butyric acid in the anticancer activity of acyloxyalkyl esters. *J. Med. Chem. 48*, 1042–1054.

(35) Trocho, C., Pardo, R., Rafecas, I., Virgili, J., Remesar, X., Fernandez-Lopez, J. A., and Alemany, M. (1998) Formaldehyde derived from dietary aspartame binds to tissue components in vivo. *Life Sci. 63*, 337–349.

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